J. Phytopathology **155**, 526–530 (2007)
doi: 10.1111/j.1439-0434.2007.01271.x

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Identification and Regulation of Genes from a Biocontrol Strain of *Fusarium oxysporum*

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Received December 29, 2006; accepted March 12, 2007

Keywords: *Fusarium* wilt, *Fusarium oxysporum*, ABC transporter, thiram, real-time quantitative PCR

Abstract

Differential display with three time points revealed that thiram altered expression of numerous genes in the biocontrol fungus *Fusarium oxysporum* CS-20. Of the 101 bands purified from the differential display gel, 86 were successfully cloned, and 64 sequenced. Based on nucleic acid sequences, homology to known products was found using BLASTn for 26 sequences and homology to hypothetical proteins was found for six sequences, also from *Gibberella zeae*. One band (BM1 24-1) showed homology to an ABC transporter from three different fungi. Because of its association with detoxification functions, the ABC transporter was selected for further study. Mycelia of CS-20 were exposed to 25 μg active ingredient (a.i.) thiram in liquid culture for various times from 0 to 8 h. Quantitative real-time PCR was used to evaluate gene expression. At 30 min after treatment with thiram, the ABC transporter was upregulated 20- to 25-fold relative to the control treatment. The ABC transporter was upregulated 15-fold at 1 h after treatment and 10-fold at 2 h. At 8 h after treatment, there was no difference between treated and non-treated for expression of the ABC transporter. Transcription of the gene encoding EST BM1 24-1 is induced in response to thiram treatment and may function in providing resistance in *F. oxysporum* isolate CS-20 to fungicides and other toxins. Tolerance to toxins may be critical to the successful inclusion of CS-20 in disease control strategies in cropping systems.

Introduction

Fusarium oxysporum is a soil-inhabiting fungus. Most literature on the identification and function of genes in the genus *Fusarium* has focused on plant pathogenic strains (e.g., Kistler, 1997; Baayen et al., 1998; Recorbet et al., 2003), including assembling an entire genome [*F. graminearum* Sequencing Project, Broad Institute of MIT and Harvard (http://www.broad.mit.edu)]. Not all strains of *F. oxysporum* are pathogenic, and among the saprophytic strains are those which act as biocontrol agents against *Fusarium* wilt (Fravel et al., 2003a). As biocontrol strains of *F. oxysporum* and other saprophytic *F. oxysporum* are morphologically identical to the pathogen, the trait or traits that confer biocontrol ability must be the result of either genetic differences between biocontrol and other *F. oxysporum*, or differences in expression of genes that may be present in both biocontrol and non-biocontrol strains.

Fusarium oxysporum CS-20 is a biocontrol strain that reduces incidence of *Fusarium* wilt on tomato (Larkin and Fravel, 1999a), muskmelon (Larkin et al., 1999), and basil (Larkin and Fravel, 2002) via a host-mediated response (Larkin and Fravel, 1999b). Preliminary work indicated that one possible difference between two biocontrol strains (including CS-20) and two pathogenic strains of *F. oxysporum* is in the expression of an ATP-binding cassette (ABC transporter) (Fravel et al., 2003b). In nature, ABC transporters provide protection against toxic compounds such as antibiotics and fungicides (Del Sorbo et al., 2000; Schoonbeek et al., 2002; Stergiopoulos et al., 2002). While strain CS-20 reduced *Fusarium* wilt under production conditions in several hosts as noted above, failure to achieve biocontrol in the field was possibly associated with the presence of a seed-applied fungicide (Fravel et al., 2005). Hence, genes involved in detoxification of fungicides in CS-20 are of interest from an ecological fitness perspective and for integrating biocontrol with fungicide use in current agricultural practices.

This study was undertaken to identify genes expressed in the *F. oxysporum* biocontrol strain CS-20 and to study the effects of the fungicide thiram on expression of an ABC transporter in CS-20.

Materials and Methods

Cultures and growth conditions

Starter cultures were prepared by inoculating 100 ml of potato dextrose broth (PDB; Difco, Sparks, MD, USA) in 250 Erlenmeyer flasks with 0.5-cm-diameter agar discs of *F. oxysporum* strain CS-20. Cultures were grown for 5 days with gentle shaking (approximately 70 rpm) under ambient conditions. Cultures were quickly vacuum-filtered through sterile Miracloth (Calbiochem, LaJolla, CA, USA) and the wet mycelia from a single starter flask were added to a new flask with the appropriate treatment. Treatments consisted of PDB alone (control), PDB with thiram [25 μg/ml active ingredient (a.i.)] (Fravel et al., 2005), or PDB with 10 μg/ml tomatine (Zwiers and De Waard, 2000). Previous studies demonstrated the toxicity of the fungicide thiram (Gustafson, Plano, TX, USA) to strain CS-20 (Fravel et al., 2005). Both thiram and tomatine (Sigma, St Louis, MO, USA) were dissolved directly in PDB. Samples were taken 2, 8 and 24 h after transfer to the treatment flasks by filtering through sterile Miracloth and immediately freezing in liquid N. Samples were stored at −80°C until use. There was one flask per treatment per time and a PDB control flask for each time point.

RNA isolation and differential display

Total RNA was extracted from mycelia using the RNeasy Mini Plant Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions, except that the RNA was eluted twice with 50 μl. A volume of 100 μl was then reapplied to the membrane and eluted a third time. The resulting RNA was then treated with DNase (Ambion, Austin, TX, USA) to remove any genomic DNA contamination.

Differential display was carried out with RNAspectra Red kit 1 (R501) (GenHunter, Nashville, TN, USA) following the manufacturer's directions. Briefly, for reverse transcription, a 20-μl reaction mixture was prepared with an H-T_{11}-anchored primer (G,A,C), total RNA, dH_2O, RT buffer and dNTP. For the PCR reaction, an H-AP arbitrary primer (1–8) and an H-T_{11}-labelled anchored primer were used. The PCR product (3.5 μl) was run on a 6% denaturing polyacrylamide gel with 2 μl FDD (99% formamide; ImM EDTA, pH8; 0.009% xylene cyanole FF; 0.009% bromophenol blue) loading dye. The gel was run on a Life Technologies Model S2 gel electrophoresis sequencing apparatus (Gaithersburg, MD) for 2–3 h at 60 W constant power. The gel was visualized using an Amersham Biosciences Typhoon 8600 Variable Mode Imager (GE Healthcare, Piscataway, NJ, USA) with a 532 green laser, and a Cy3, 555, BP20 emission filter. Bands that were different between the PDB control treatment and the thiram or tomatine treatment were selected, excised and eluted from the gel, and reamplified following the procedure described in the GenHunter protocol. The reamplified cDNA fragments were cloned into *Escherichia coli* using the PCR-TRAP Cloning System (GenHunter). Following successful cloning, the products were sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The manufacturer's instructions were followed for the labelling reaction and the reaction purification, and the samples were sequenced on an ABI Prism 3700 (Applied Biosystems). Each cloned DNA product was sequenced at USDA (ARS, Beltsville, MD) at least twice in each direction. Sequence data were assembled using the default settings in Seqman II (DNAstar, Madison, WI). Vector sequence contamination was removed and the resulting sequences were identified using BLASTn.

Real-time quantitative PCR (QPCR)

To further understand the expression of the ABC transporter identified above (Table 1), a more thorough time course was established. Starter cultures of strain CS-20 were again grown in PDB for 5 days before transfer to treatment flasks. Samples were taken immediately after filtering through Miracloth but before placing in treatment flasks, as well as at 0, 0.5, 1, 2 and 8 h after placement in treatment flasks. Immediately after filtering, all samples were frozen in liquid N then stored at −80°C until use. There was one replicate flask per treatment per time and the experiment was conducted three times. Mycelia from additional flasks were weighed after filtering but immediately prior to placement in treatment flasks as well as at the conclusion of the experiment to determine if treatments affected growth of mycelia.

RNA was extracted with RNeasy Mini Plant Kit (Qiagen) as described above and then treated with DNAfree (Ambion) to remove any contaminating gDNA. All RNA samples were run on an agarose gel to verify RNA integrity. The RNA was quantified on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

The RNA was reverse-transcribed into cDNA with an iScript kit (Bio-Rad, Hercules, CA, USA), and subjected to quantitative PCR (QPCR) on an iCycler (Bio-Rad). Primers for the identified DNA sequences were selected with Beacon Designer (Bio-Rad) and were prepared by Integrated DNA Technologies (Coralville, IA, USA). Primers used were BM1 241F (5′-TGGTCAATCCGCTTGTCCTCACTA-3′) and BM1 241R (5′-TGCGTAAGCCACTCCTTGAGGTAT-3′). The PCR cocktail comprised of 10 μl SYBR Green Supermix (Bio-Rad), 7 μl of H_2O, 1 μl of each primer (2 μM), and 1 μl of cDNA. PCR consisted of 95°C for 3 min followed by 40 cycles of 95°C for 10 s then 63°C for 45 s. The constitutively expressed *Gibberella zeae* (*Fusarium graminearum*) *ACTIN* gene (XM_390561) was used as an expression control. Data were analysed using the BioRad Gene Expression Analysis Macro for Microsoft Excel. PCR for each replicate flask (time point) was conducted at least three times and these were considered subsamples. Treatments were compared using analysis of variance (SAS, Cary, NC, USA).

Table 1
Homology of sequences from the biocontrol fungus *Fusarium oxysporum* strain CS-20 with sequences using BLASTn in the NCBI GenBank database

Sequence[a]	Accession number[b]	Product	Size (BP)	Organism	E-value
BM1 24-1	XM_389006	cd00267: ABC_ATPase; ABC (ATP-binding cassette) transporter nucleotide-binding domain	650	*Gibberella zeae* PH-1	5.00E-35
BM1 24-1	XM_655841	ABC (ATP-binding cassette) transporter nucleotide-binding domain	650	*Aspergillus nidulans*	9.00E-06
BM1 24-1	AY911669	ABC transporter	650	*Trichoderma atroviride*	6.00E-04
BM1 6-1	XM_389185	Ribonuclease III C terminal domain	591	*Gibberella zeae* PH-1	9.00E-18
BM1 9-1	XM_382457	C4-dicarboxylate transporter/malic acid transport protein	500	*Gibberella zeae* PH-1	2.00E-34
BM2 19-5	AL132949	Cyclic nucleotide phosphodiesterases	220	*Caenorhabditis elegans*	6.00E-04
BM2 22-1	XM_386364	Hypothetical protein	395	*Gibberella zeae* PH-1	5.00E-40
BM2 25-2	AB187268	18S rRNA	307	*Cordyceps sinensis*	7.00E-08
BM2 25-3	AB187268	18S rRNA	303	*Cordyceps sinensis*	7.00E-08
BM2 25-4	XM_391031	Unknown function	211	*Gibberella zeae* PH-1	2.00E-102
BM2 37-1	XM_386751	CCC1-like, a yeast vacuole transmembrane protein	731	*Gibberella zeae* PH-1	4.00E-116
BM2 37-2	XM_380312	Transmembrane amino acid transporter protein	415	*Gibberella zeae* PH-1	3.00E-56
BM4 10-1	XM_389185	Ribonuclease III C terminal domain	458	*Gibberella zeae* PH-1	7.00E-18
BM4 10-2	XM_386465	Ribosomal protein L3	407	*Gibberella zeae* PH-1	2.00E-89
BM4 10-3	XM_385405	Bystin	364	*Gibberella zeae* PH-1	1.00E-34
BM4 12-1	XM_381584	FKBP-type peptidyl-prolyl cis-trans isomerase	378	*Gibberella zeae* PH-1	1.00E-90
BM4 12-2	XM_388525	GAL4-like Zn2Cys6 binuclear cluster DNA-binding domain	342	*Gibberella zeae* PH-1	3.00E-38
BM4 12-3	XM_382938	Ribosomal protein S11	310	*Gibberella zeae* PH-1	4.00E-40
BM4 41-1	XM_380668	Hypothetical protein	241	*Gibberella zeae* PH-1	9.00E-16
BM4 41-2	XM_385225	Ribosomal RNA adenine dimethylases	316	*Gibberella zeae* PH-1	7.00E-05
BM4 8-1	XM_381508	Nucleic-acid-binding protein	315	*Gibberella zeae* PH-1	5.00E-21
BM5 42-4	XM_382253	Hypothetical protein	467	*Gibberella zeae* PH-1	5.00E-19
BM5 6-3	XM_388726	Ankyrin repeats	481	*Gibberella zeae* PH-1	8.00E-55
BM5 6-4	XM_382457	C4-dicarboxylate transporter/malic acid transport protein	495	*Gibberella zeae* PH-1	1.00E-24
BM5 6-5	XM_386987	Hypothetical protein	510	*Gibberella zeae* PH-1	4.00E-07
BM5 6-9	XM_388623	WD40 domain	251	*Gibberella zeae* PH-1	3.00E-25
BM6 22-1	XM_387393	Dihydrouridine synthase (Dus)	311	*Gibberella zeae* PH-1	4.00E-06
BM6 34-3	XM_390916	Microtubule associated protein 1A/1B	369	*Gibberella zeae* PH-1	2.00E-48
BM6 4-1	XM_387393	Dihydrouridine synthase (Dus)	301	*Gibberella zeae* PH-1	4.00E-06
MS47 10-3	XM_386500	GAL4-like Zn2Cys6 binuclear cluster DNA-binding domain	337	*Gibberella zeae* PH-1	8.00E-11
MS47 10-4	XM_386500	GAL4-like Zn2Cys6 binuclear cluster DNA-binding domain	187	*Gibberella zeae* PH-1	4.00E-11
MS47 10-7	XM_381749	Aminotransferase class-III	182	*Gibberella zeae* PH-1	7.00E-16
MS47 3-1	XM_380668	Hypothetical protein	717	*Gibberella zeae* PH-1	4.00E-14

[a]Sequences from *F. oxysporum* CS-20 with GenBank EST database accession numbers DW177382 to DW177412.
[b]Accession numbers from organisms in column five of this table.

Gene copy number

The number of copies the gene BM1 24-1 in *F. oxysporum* strains CS-20 was determined by Southern blot. Fungal DNA was extracted with DNeasy Mini Plant Kit (Qiagen) following the manufacturer's instructions; 16 μl of the resulting total DNA was cut with each of the following restriction enzymes: *Eco*RI, *Bgl*II, *Hin*dIII, and *Xho*I; 30 μl of the cut DNA was run on a 0.8% agarose gel for 1.5 h at 85 V. After photographing, the gel was transferred to a nitrile membrane using standard alkaline transfer technique.

The DNA probe for BM1 24-1 was produced using PCR and the primers mentioned above (BM1 24-1F and BM1 24-1R). Bands from this PCR were excised from a gel and purified. The purified probe DNA fragment was labelled with Fluorescen 12-dutp using the Random primer labelling kit from Enzo (42726, Farmingdale, NY). Enzo protocols for hybridization and detection were used.

Results

Band isolation, cloning and identification

Differential display with three time points revealed that both thiram and tomatine altered expression of numerous genes. Bands of genes that appeared to be increased or decreased in expression compared with the PDB treatment were excised from the sequencing gel (101 bands) and cloned into *E. coli*. Eighty-six bands were successfully cloned and 64 were sequenced. When sequences were compared with known sequences (BLASTn), no homology was found to 32 sequences and probable functions could be assigned to 26 sequences (Table 1). Most bands listed in Table 1 showed homology to those from *Gibberella zeae*. One band (BM1 24-1) showed homology to an ABC transporter from three different fungi.

Regulation of ABC transporter (BM1 24-1)

Because of its association with detoxification functions and prior indication that expression might be different between pathogenic and biocontrol *F. oxysporum* strains (Fravel et al., 2003a,b), an ABC transporter was selected for further study.

In the presence of thiram, fresh weight of mycelia was significantly reduced by 32% ($P \leq 0.05$) over the 8-h experiment. In response to thiram, the gene encoding the ABC transporter (BM1 24-1) was significantly upregulated 19- to 24-fold relative to the PDB control treatment at 30 min after exposure, and upregulated 14.5- and 7-fold after 1 and 2 h, respectively ($P \leq 0.05$; Fig. 1). After 8 h, expression of the ABC transporter was not different from that of the PDB control treatment. Samples taken just prior to the start of the experiment indicated that handling the mycelia to the extent needed for the experiment did not alter regulation of the ABC transporter.

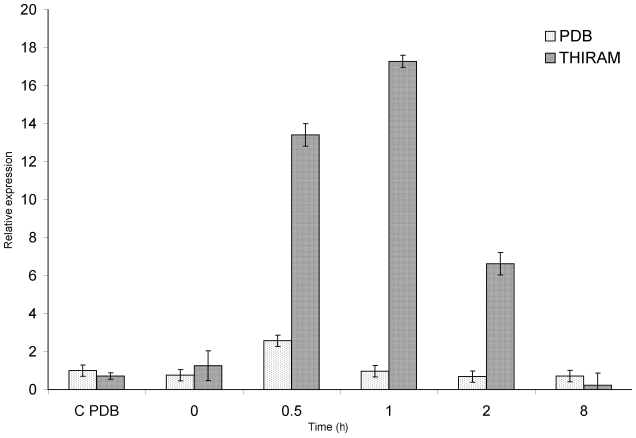

Fig. 1 Expression of an ABC transporter (BM1 24-1) in the biocontrol fungus *Fusarium oxysporum* CS-20 in response to the presence or absence of 25 μg a.i./ml of the fungicide thiram. C PDA denotes sampling of starter cultures in potato dextrose broth immediately prior to beginning the experiment. Bars represent standard deviation of the mean ($P \leq 0.05$)

Gene copy number

Banding patterns of the southern blot cut with four different restriction enzymes indicated that strain CS-20 has three copies of BM1 24-1.

Discussion

Transcription of BM1 24-1 is responsive to the fungicide thiram and putatively encodes an ABC transporter (Table 1). In nature, ATP-binding cassettes provide protection against toxic compounds such as antibiotics and fungicides, as well as against phytoalexins (Andrade et al., 2000; Del Sorbo et al., 2000; Schoonbeek et al., 2001, 2002; Stergiopoulos et al., 2002; Zwiers et al., 2003). It is not currently known whether ABC transporters are involved in biocontrol ability but could allow the establishment of biocontrol organisms within plant tissues by providing tolerance to phytoalexins. Mycotoxin detoxification could also confer competitive ability against plant pathogenic fusaria (Lutz et al., 2003) and other microbes. ABC transporters are known to play a role in the pathogenicity of several fungi including pathogenicity of wheat and barley by *F. culmorum* (Skov et al., 2004). Differences in fungal virulence have been attributed to differences in gene expression, including differences of unidentified genes in *F. graminearum* (Goswami et al., 2002).

Fusarium oxysporum is a large and complex species. While the genetic and biochemical pathways involved in pathogenesis are considered poorly understood (Recorbet et al., 2003), even less is known about the genetic and biochemical determinants of successful biocontrol of *Fusarium* wilt by nonpathogenic *F. oxysporum*. This research was undertaken as an initial step towards understanding these determinants. More knowledge of the ABC transporter in strain CS-20 could provide insight into integrating this biocontrol into a production system with fungicides toxic to

CS-20 or into selecting new strains that would be more compatible with fungicides. Additional research is needed to determine whether regulation of this ABC transporter differs among biocontrol, non-biocontrol and pathogenic *F. oxysporum*.

Acknowledgements

The authors thank Mary Strem and Chalita Atallah for technical assistance.

Conflict of Interest

Mention of trade names or commercial products in this article is solely for the purpose of providing scientific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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